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**KPC-2-producing *Klebsiella pneumoniae* ST147 in a neonatal unit: Clonal isolates with differences in colistin susceptibility attributed to AcrAB-TolC pump.**

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28    **Running title:** Differential colistin susceptibility in clonal *K.pneumoniae*

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40 **Abstract:-**

41 This study describes the characterization of four KPC-2-producing *Klebsiella pneumoniae* strains  
42 from neonates belonging to a single sequence type 147 (ST147) in relation to carbapenem resistance  
43 and explores probable mechanisms of differential colistin resistance among the clonal cluster.

44 Whole Genome Sequencing revealed that the isolates were nearly 100% identical harbouring  
45 resistance genes (*bla<sub>KPC-2</sub>*, OXA-9, CTX-M-15, SHV-11, OXA-1, TEM-1B, *oqx<sub>A</sub>*, *oqx<sub>B</sub>*, *qnrB1*, *fosA*, *arr-2*, *sul1*,  
46 *aacA4*, *aac(6')Ib-cr*, *aac(6')Ib*), and several virulence genes. *bla<sub>KPC-2</sub>* was the only carbapenem-  
47 resistant gene found, bracketed between *ISKpn7* and *ISKpn6* of Tn4401b on a non-conjugative  
48 IncFII plasmid. Remarkably, one of the clonal isolates was resistant to colistin, the mechanistic basis  
49 of which was not apparent from comparative genomics. The transmissible colistin resistance gene,  
50 *mcr*, was absent. Efflux pump inhibitor CCCP rendered 32-fold decrease in the minimum inhibitory  
51 concentration of colistin in the resistant isolate only. *acrB*, *tolC*, *ramA*, and *soxS* genes of the  
52 AcrAB-TolC pump system overexpressed exclusively in the colistin-resistant isolate, although the  
53 corresponding homologs of AcrAB-TolC pump, regulators and promoters were mutually identical.  
54 No change was observed in the expression of other efflux genes (KpnEF & KpnGH) or Two  
55 Component System genes (*phoP/phoQ*, *pmrA/pmrB*).

56 We postulate that colistin resistance in one of the clonal KPC-2-producing isolate was due to  
57 overexpression of AcrAB-TolC pump. This study is probably the first to report clinical clonal *K.*  
58 *pneumoniae* isolates with differences in colistin susceptibility. The presence of carbapenem-resistant  
59 isolates with differential behavior in the expression of genomically identical pump system indicates  
60 the nuances of the resistance mechanisms and the difficulty of treatment thereof.

61 **Keywords:** KPC-2, *Klebsiella pneumoniae*, ST147, Colistin resistance, AcrAB-TolC pump, Two  
62 Component System.

## 63    **1. Introduction: -**

64    Carbapenem resistance has challenged the medical system all around the globe. The repertoire of  
65    carbapenem-resistant genes (class A, B and D enzymes) and their spread via mobile genetic  
66    elements have compounded the problem. *Klebsiella pneumoniae* carbapenemases (KPCs), class A  
67    enzymes, confer decreased susceptibility to virtually all  $\beta$ -lactams and are mostly present in  
68    *Klebsiella pneumoniae* [1,2]. *K. pneumoniae* causes a wide range of infections, such as pneumonia,  
69    urinary tract infections, septicemia, liver abscesses etc., not only in immunocompromised as well as  
70    in immunosufficient ones [3]. According to the resistance map of CDDEP reports (2017) for India,  
71    59% of *K. pneumoniae* are resistant to carbapenems, and this has increased from 56% in 2015  
72    (<https://resistancemap.cddep.org/>).

73            Till now 46 types of KPCs have been reported worldwide  
74    (<https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/KPC>). Though, the spread of KPC had  
75    been initiated by a single dominant clone sequence type 258 (ST258), it has now been reported  
76    among varied STs across the world [1,2].

77            With increased resistance towards carbapenems, colistin is being used as one of the last  
78    treatment options [1]. Isolates which are resistant to both colistin and carbapenem have been  
79    reported [4]. Such isolates are dreaded in clinical setups, particularly in neonatal intensive care units  
80    (NICU) as treatment options are already limited in the newborns. Colistin being a cationic peptide,  
81    acts by disrupting the negatively charged lipopolysaccharide (LPS). Resistance to colistin is  
82    generally attributed to upregulation of two-component system genes (TCSs) such as *phoP/phoQ* and  
83    *pmrA/pmrB*. Constitutive activation of these genes leads to modification of LPS, leaving colistin  
84    ineffective. Another important mechanism of resistance is by mutations/inactivation of *mgrB*, a

transmembrane protein, acting as a negative feedback loop for *phoP/phoQ* system. Nevertheless, many other mechanisms work simultaneously resulting in colistin resistance. With the emergence of mobilized colistin resistance gene, *mcr*, spread of resistance has increased several folds [4–6].

This study characterizes four clonal *K. pneumoniae* collected from a NICU in terms of carbapenem resistance. The clonal isolates also showed differences in resistance to colistin. Given the fact that such difference in colistin resistance in genetically identical carbapenem-resistant isolates is rarely observed, and analysis of the mechanism of colistin resistance among these isolates was also carried out.

## **2. Materials and Methods: -**

### ***2.1. Identification of bacterial isolates, antimicrobial susceptibility testing and phenotypic assay for Extended Spectrum Beta-Lactamases (ESBLs) and Carbapenemases:***

Bacterial isolates were collected from the blood of septicemic neonates from the neonatal intensive care unit of a tertiary care hospital of Kolkata, West Bengal by standard procedure. Isolation was done from the year 2013-2016, in which a total of 195 Enterobacteriaceae were recovered. Isolates were identified by in-house biochemical tests and further confirmed by VITEK® 2 Compact system (bioMérieux, Marcy-l'Étoile, France). Minimum inhibitory concentrations (MICs) were determined by E-test method (bioMérieux, Marcy-l'Étoile, France) for different antimicrobial agents. For colistin and fosfomycin (MP Biomedicals, California, USA), broth micro dilution and agar dilution were carried out respectively [7]. Results were analyzed according to Clinical & Laboratory Standards Institute (CLSI) for all antibiotics except for colistin & tigecycline which were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Since the breakpoint of tigecycline was not available for *K. pneumoniae* in EUCAST, thus the

breakpoint for *Escherichia coli* in EUCAST was used for *K. pneumoniae* (susceptible  $\leq 0.5$  mg/L; resistant  $> 0.5$  mg/L) [8,9].

Isolates were tested for the production of ESBLs and carbapenemases with the ROSCO tablets according to manufacturer's instruction. Isolates exhibiting an increase of  $\geq 5$  mm in the inhibition zone of the combination disc with respective antibiotics were categorized as positive. [Rosco Diagnostica A/S, Taastrup, Denmark].

### 2.2. Genotypic determinants in *K. pneumoniae* isolates:

Based on the susceptibility pattern and phenotypic results, polymerase chain reaction (PCR) was done for the following  $\beta$ -lactamase genes (*bla*<sub>CTX-M,TEM,SHV,OXA-1</sub>), AmpC genes (*bla*<sub>MOX,CMY,DHA,ACC,MIR/ACT,FOX</sub>), carbapenemase genes (*bla*<sub>VIM,IMP,SPM-1,GIM-1,SIM-1,NDM-1</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>KPC,SME,IMI,GES,NMC</sub>), 16S rRNA methylase encoding genes (*rmtA*, *rmtB*, *rmtC*, *rmtD* & *armA*), flouroquinolone genes (*qnr-A,B,S*, *qepA*, *aac(6')-Ib*, *aac(6')-Ib-cr*), addiction systems and virulence genes as described previously [10]. Plasmid types of the isolates were identified by PCR based replicon typing (PBRT) [11].

Primer walking was done to discern the genetic environment of the carbapenemase genes [2]. Primer sequences have been enlisted in Table S1.

### 2.3. Whole genome sequencing (WGS):

Total genomic DNA was extracted using the QIAamp DNA kit, and quantified using the Qubit 3.0 (Thermo Fisher Scientific). DNA libraries were prepared for paired end sequencing (2x301 cycles) using Nextera XT (Illumina Inc., San Diego, CA). Whole genome sequencing (WGS) was performed using the v3 chemistry on the Illumina MiSeq platform (Illumina Inc., San Diego, CA).

128           Quality control of raw reads included fastqc (0.11.2), and quality and adaptor trimming was  
129 performed using Trimgalore (0.4.3). Reads were assembled in contigs using the *de novo* assembler  
130 SPAdes (3.9.0) (.fasta) and were aligned to the original fastq reads using BWA aligner (0.7.15). Any  
131 assembly mapping errors in the contigs was corrected using Pilon (1.22). Assembly metrics were  
132 evaluated using Quast (2.1). Genomes (contigs) were annotated using Prokka (1.12) and the resulting  
133 .gbk file was viewed in Artemis (genome viewer, Sanger, UK). Resulting .gff files of Prokka were  
134 used to create a core genome alignment using Roary (v3.12.0). Fasttree was used to build the  
135 phylogenetic tree (.nwk file) and visualized in Interactive Tree Of Life (iTOL). BLAST Ring Image  
136 Generator (BRIG) [12] was used to evaluate genome wide sequence similarity using a KPC-2-  
137 producing ST11 *Klebsiella pneumoniae subsp. pneumoniae HS11286* as a reference genome. Single  
138 nucleotide polymorphism (SNP) analysis was done using kSNP3.0 using the file generated with  
139 Prokka [13].

140           ResFinder (3.0), PlasmidFinder (1.3) were used for genotypic characterization. Virulence  
141 genes were assessed by Institut Pasteur MLST and whole genome MLST databases  
142 (<http://bigsd.b.pasteur.fr/klebsiella/klebsiella.html>) as well as by Kleborate pipeline  
143 (<https://github.com/katholt/Kleborate>) for more detailed analysis of the isolates. Promoter sequence  
144 analysis was done ([http://www.fruitfly.org/seq\\_tools/promoter.html](http://www.fruitfly.org/seq_tools/promoter.html)). Individual genes were BLAST  
145 searched against the four genomes along with MUMmer alignment. Genomes were visualized using  
146 SNAP Gene tool (4.3.8). INTEGRALL database (<http://integrall.bio.ua.pt>) was used to search the  
147 integron sequences. TETyper (<https://omictools.com/tetyper-tool>) was used with the suggested  
148 parameters, to type the transposable genetic element from paired-end sequencing data.

149   **2.4. Multi-locus Sequence Typing (MLST) and Pulse Field Gel Electrophoresis (PFGE):**



150 To identify the sequence types (ST), MLST database (<https://cge.cbs.dtu.dk/services/MLST/>) was  
151 used.

152 PFGE was performed using PulseNet standardized procedures with *XbaI* (50 U/plug)  
153 (<http://www.cdc.gov/pulsenet/protocols.html>) in a CHEF-DR III system (Bio-Rad Laboratories,  
154 Hercules, CA, USA). *XbaI* macrorestriction patterns were visually interpreted according to Tenover  
155 criteria [14].

## 156 ***2.5. Transmissibility of carbapenemase carrying plasmid:***

157 The transfer of carbapenemase gene into *E. coli* J53Az<sup>r</sup> strain was performed by both solid- and  
158 liquid-mating conjugation techniques at varying temperatures. Electroporation was carried out with  
159 purified plasmid DNA [15] into *E. coli* DH10B (Invitrogen, California, USA) using a Gene Pulser II  
160 (Bio-Rad Laboratories, Hercules, CA, USA). Transformants were selected on LB agar plates (BD  
161 BBL, MD, USA) supplemented with ertapenem (0.25mg/L) (Sigma-Aldrich, St Louis, MO, USA).  
162 Transformants retrieved were subjected to genotypic confirmation of carbapenemase and other  $\beta$ -  
163 lactamase genes followed by susceptibility testing.

## 164 ***2.6. Evaluating mechanisms of colistin resistance -***

165 WGS data was evaluated using ResFinder for the presence of *mcr* or its variant genes.

### 166 ***2.6.1. Efflux pump activity:***

167 MIC of colistin was determined in the presence of efflux pump inhibitors (EPIs) such as carbonyl  
168 cyanide 3-chlorophenylhydrazone (CCCP; 10mg/L) and 1-(1-naphthylmethyl)-piperazine (NMP;  
169 25mg/L) (Sigma-Aldrich, St Louis, MO, USA) to assess the role of efflux pump genes [16, 17]. MIC  
170 was done in triplicate in separate experiments.

### 171 ***2.6.2. Gene expression using real-time PCR (RT-PCR):***

172 The mRNA expression level of TCS genes viz *phoP/phoQ*, *pmrA/pmrB* were analyzed [4,5]. Based  
173 on the phenotypic expression of EPIs, expression of transmembrane protein of AcrAB-TolC (*acrB*),  
174 *acrA*, *tolC* and their regulators (*soxS* and *ramA*) were studied [18]. Additionally, expression of  
175 KpnEF (small multidrug resistance family, SMR) and KpnGH (Major facilitator superfamily, MFS)  
176 efflux pumps [19, 20] were also analyzed.

177 Isolates were inoculated overnight in cation-adjusted Mueller-Hinton broth (BD BBL, MD,  
178 USA) in the presence of colistin sulphate (0.125mg/L) at 37°C with shaking. Overnight cultures  
179 were diluted (1/100) in cation-adjusted Mueller–Hinton broth containing colistin sulphate  
180 (0.125mg/L) and grown to mid-logarithmic phase at 37°C with vigorous shaking (230 rpm). RNA  
181 was harvested using NucleoSpin® RNA purification kit (Machery-Nagel, Germany) according to the  
182 manufacturer's instructions. RNA yield and quality were determined using a NanoDrop  
183 biophotometer plus (Eppendorf, Hamburg, Germany) followed by RNase-free DNase treatment  
184 (New England Biolabs, USA) and purity was assessed by PCR.

185 Two micrograms of the isolated total RNA were reverse transcribed to cDNA using the  
186 RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, Massachusetts, USA).  
187 Expression of the target genes were quantified using Power SYBR Green PCR Master Mix (Applied  
188 Biosystems, California, USA) in StepOne Plus Real-Time PCR system (Applied Biosystems,  
189 California, USA). The reactions were performed with 0.5 mM primer in 20 µL reaction volumes in  
190 duplicate in separate experiments. The relative RNA expression levels were calculated according to  
191 the  $\Delta\Delta C_t$  method, normalized with the ribosomal housekeeping genes (*rpsL* for TCS, KpnEF,  
192 KpnGH pumps, and *rrsE* for AcrAB pump). Relative expression of each target gene was then  
193 calibrated against the expression of a *K. pneumoniae* (EN5219) isolate susceptible to all antibiotics  
194 tested, which served as the reference strain (expression=1). A  $\geq 2$ -fold increase in the expression of

195 genes when compared with the reference strain was considered as cut-off to denote significant  
196 overexpression [21].

### 197 **2.6.3. Outer membrane protein profile:**

198 Whole-cell extracts of the isolates were separated on 12% SDS–polyacrylamide gels and transferred  
199 to Immobilon-P membrane (Merck Millipore, Massachusetts, USA) following standard protocols.  
200 An isolate of *E. coli* (S205) which retained both the outer membrane proteins (OMPs) (OmpC/F)  
201 was used as a control for the western blots. Porins were detected using polyclonal anti-OmpC/F  
202 antibody with 1:3000 dilutions as described earlier [22].

## 203 **3. Results and Discussion:**

### 204 **3.1. Identification of isolates and clinical presentation of neonates:**

205 Of the different Enterobacteriaceae evaluated during the period of 2013-2016, only four isolates  
206 possessed *bla*<sub>KPC</sub> and all four were *K. pneumoniae*. These four isolates were recovered during  
207 September, 2015 to December, 2015 indicating the emergence of this gene in this unit. There were  
208 no further cases of *bla*<sub>KPC</sub>-harbouring isolates till end of 2016. The four isolates were named as *Kp1*  
209 (EN5226), *Kp2* (EN5261), *Kp3* (EN5269), and *Kp4* (EN5271) and analyzed in detail. Henceforth,  
210 isolates will be referred to as *Kp1*, *Kp2*, *Kp3* and *Kp4* isolated from neonates P1, P2, P3, and P4  
211 respectively.

212 The details of the clinical status of the neonates are presented in Table S2. P1 had an  
213 extended stay of nearly a year and P2 was admitted for 2 months. P3 and P4 were in the unit for  
214 almost a month (Table S2). Since *bla*<sub>KPC</sub> was never recovered from this unit before or after the  
215 aforesaid period, hence P1 carrying the *bla*<sub>KPC</sub> was suspected to be the index case and all neonates  
216 had overlapping stay, indicating possibility of cross transmission. None of the neonates were treated

217 with colistin during their stay. Other studies have also reported nosocomial outbreaks [23] with  
218 KPC-producing strains including outbreaks in neonatal units [24].

### 219 **3.2. Antibiotic susceptibility testing and phenotypic detection of ESBLs and carbapenemases:**

220 Antibiotic susceptibility tests for the four isolates showed similar pattern of susceptibility for 14  
221 antimicrobials except for colistin. *Kp1*, *Kp3*, and *Kp4* were susceptible to colistin whereas *Kp2* was  
222 resistant (MIC: 8mg/L). *Kp1-Kp4* was susceptible to gentamicin, ceftazidime, meropenem, and  
223 tigecycline only (Table 1).

224 ROSCO combination tablets indicated presence of ESBLs and KPC which were further  
225 verified by PCR/WGS.

### 226 **3.3. Resistance and virulence determinants of *K. pneumoniae*:**

227 Resistome analysis ( $\geq 98\%$  identity and coverage) showed that the four strains possessed *bla*<sub>KPC-2</sub> as  
228 the only carbapenemase gene apart from the following genes: 5  $\beta$ -lactamase genes *bla*<sub>OXA-9</sub>, *bla*<sub>CTX-M-15</sub>,  
229 *bla*<sub>SHV-11</sub>, *bla*<sub>OXA-1</sub>, and *bla*<sub>TEM-1B</sub>; *aacA4*, *aac(6')Ib* and *aadA1* (aminoglycoside resistance-  
230 encoding genes); *aac(6')Ib-cr*, *qnrB1*, *oqxA*, and *oqxB* (quinolone resistance); *fosA* for fosfomycin;  
231 *catB3* for phenicol; *arr-2* for rifampicin and *sulI* for sulphonamide (Table 2). Resistance  
232 determinants mentioned above were also detected by conventional PCRs to validate the results  
233 obtained from WGS analysis. *vagCD* was the only addiction system found in these isolates (Table  
234 1). In accordance to earlier reports, our study also revealed association of KPC with resistance  
235 determinants conferring resistance to other antibiotics [1]. KPC (serine  $\beta$ -lactamases) are efficient in  
236 hydrolyzing cephalosporins, monobactams, and carbapenems (imipenem, and ertapenem). KPCs  
237 often evade detection by routine susceptibility screening as they are not as efficient as other  
238 carbapenemases, hence, leading to treatment failures and possess an exceptional potential for  
239 dissemination [1].

240 Different virulence genes, such as *fimH* (type-1 fimbrial adhesion protein), *wabG* & *uge*  
241 (biosynthesis of the outer core lipopolysaccharide; capsule and smooth lipopolysaccharide  
242 respectively), *kfuBC* (iron-uptake system), *ybt* (siderophore yersiniabactin cluster), *mrk* (type-3  
243 fimbrial gene cluster), and *fyuA* (yersiniabactin receptor) were found within the genomes of the four  
244 isolates. Presence of these genes were also reported in *K. pneumoniae* from clinical setups [25, 26].  
245 Isolates in this study harbored KL10 (K-loci), O3/O3a (O-loci) bearing *wiz420* and *wcz11* capsular  
246 genes. All the four strains belonged to yersiniabactin sequence type 135 (ybST135) and carried  
247 ybt14; ICEKp5 as the integrative conjugative element (Table 2). These virulence determinants  
248 provide the isolates with both adaptive advantages and offensive capabilities to persist and cause  
249 disease, thereby making *K. pneumoniae* an efficient nosocomial pathogen and good colonizer.

#### 250 **3.4. Transmissibility of KPC-bearing plasmids:**

251 Transfer of KPC-2-bearing plasmid was unsuccessful by conjugation. Transformants (TF) were  
252 obtained on Luria agar plate supplemented with 0.25mg/L ertapenem upon electroporation. Presence  
253 of *bla*<sub>KPC-2</sub>, *qnrB1*, *oqxA*, *oqxB*, *aac* (6')-Ib-cr were found in *Kp2-Kp4* whereas only *bla*<sub>KPC-2</sub> was  
254 found in transformants of *Kp1* (Table 1). MICs of transformants are presented in Table 1.

255 Plasmid-scaffold genes such as IncFIB, IncFII, and IncR were found by PBRT (Table 1) and  
256 an additional Col plasmid was found by PlasmidFinder tool (Table 2). Plasmid analysis revealed  
257 presence of *bla*<sub>KPC-2</sub> on IncFII plasmid. *bla*<sub>KPC-2</sub> has been reported to reside on numerous plasmid  
258 types, such as IncF, IncI2, IncX, IncA/C, IncR, and ColE1. IncFII<sub>K</sub> replicon is the preferred one [2].  
259 IncF/ IncFII plasmids are generally conjugative [27] but in this study the IncFII plasmids were non-  
260 conjugative and could be transferred only by electroporation. This could also be a reason for its  
261 restrictive spread in the unit. Only four neonates were infected and spread of *bla*<sub>KPC-2</sub> to other species  
262 was not observed (unpublished data).

263 Genetic environment of *bla*<sub>KPC-2</sub> was determined by primer walking method which was  
264 further validated by WGS data. *bla*<sub>KPC-2</sub> was found to be within a Tn3-based transposon, Tn4401 and  
265 TETyper revealed it to be Tn4401b. It harbored two different types of IS elements. IS*Kpn7*  
266 belonging to IS21 family, in the upstream of *bla*<sub>KPC-2</sub>, and IS*Kpn6*, a member IS1182 family, on  
267 downstream of the gene. Presence of transposase, *tnpA* and resolvase, *tnpR* of Tn4401b were found  
268 at the upstream of *bla*<sub>KPC-2</sub> right before IS*Kpn7* (Fig. 1). The results corroborate with other studies  
269 reporting the association of *bla*<sub>KPC-2</sub> with Tn4401b [2]. Presence of two IS elements each having its  
270 own transposase activity along with transposase of the Tn3-based transposon, itself initiate a strong  
271 transposition event. This facilitates movement of *bla*<sub>KPC-2</sub> among various plasmids as well as  
272 different organism leading to worldwide dissemination of the gene.

273 In875 class 1 integron was found in *Kp1-Kp3*. In *Kp4*, the 3'CS region could not be retrieved,  
274 though it carried class 1 integron. In all of the isolates, insertion of *S.ma.I2* group IIc intron has been  
275 found within the *attC* site of the *arr2* gene cassette (Fig. 2).

### 276 **3.5. Molecular typing of *K. pneumoniae*:**

277 BRIG analysis revealed that the four genomes had significant levels of similarities with the reference  
278 strain (Fig. 3). Nearly 100% identity across the genomes was evident, though the four genomes also  
279 have minute differences among themselves which is common between clonal populations. The core  
280 genome alignment and resulting phylogenetic tree shows that the four isolates group in 2 clusters.  
281 *Kp2* and *Kp4* are more closely related, as are isolates *Kp1* and *Kp3* (Fig. 4A). MLST revealed that  
282 all the four isolates belonged to a single sequence type 147 (ST147) (Table 2). This finding matched  
283 with indistinguishable band patterns in PFGE (Fig. 4B).

284 Presence of *bla*<sub>KPC</sub> has been reported in almost 115 different STs worldwide of which, ST258  
285 is the leading one [2,23]. KPC-2 was found in ST258 in Europe and USA, and in ST37, ST392,  
286 ST395, and ST11 in Asia-Pacific regions [2]. In our study, *bla*<sub>KPC-2</sub> was harbored in ST147 *K.*  
287 *pneumoniae*. Interestingly, the ST147 *K. pneumoniae* clone has been associated with different  
288 carbapenemases including NDM, KPC, OXA-48, OXA-181 or OXA-204 enzymes; making this ST a  
289 globally high-risk clone [28-30]. Presence of ST147 *K. pneumoniae* has been reported from India  
290 previously in association with colistin resistance [31] but presence of *bla*<sub>KPC-2</sub> was never encountered  
291 in ST147 from India.

### 292 **3.6. Analysis of colistin resistance:**

293 Of the four clonal isolates, one (*Kp2*) demonstrated raised MIC value (8mg/L) for colistin. Clonal  
294 isolate with difference in susceptibility is rarely encountered. Though an earlier study had reported  
295 variations in susceptibility to meropenem in clonal *K. pneumoniae* [32] but to the best of our  
296 knowledge, no such variation in terms of colistin has been reported yet.

297 WGS data was extensively explored in search of recently reported *mcr* genes and its variants.  
298 No such gene was found to be present within the genome of the colistin-resistant strain. SNP  
299 analysis showed a total of 12 SNPs within the core genome of *Kp2* which differ with the other 3  
300 strains (*Kp1*, *Kp3*, *Kp4*). But none of these genes have been reported to be associated with colistin  
301 resistance. Details of the SNPs have been included in Table S3.

302 Colistin resistance is chiefly mediated by covalent modification of the lipid A moiety of  
303 lipopolysaccharide (LPS) by 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine  
304 (PEtN), resulting in reduced binding ability of colistin to the LPS [33]. Based on the established  
305 mechanisms of colistin resistance, expression of TCSs was studied by realtime RT-PCR. *phoP*,

306 *phoQ*, *pmrA*, and *pmrB* did not show significant change among the isolates when compared with  
307 reference sample EN5219 (Table 3). Sequences of these genes along with other genes known to play  
308 a significant role in colistin resistance such as *mgrB*, *pmrC*, *pmrD* (responsible for downstream  
309 modification of LPS), *crrAB* (two-component regulator of *pmrAB*), and *arnBCADTEF-pmrE*  
310 (operon that mediate synthesis and transfer of L-Ara4N to LPS) were analyzed from the WGS data  
311 but no change was observed in their sequences, which ruled out the involvement of the TCSs and  
312 other modifying genes for colistin resistance in *Kp2*.

313 A decrease in MIC of colistin from 8mg/L to 0.25mg/L (32-fold) was found in case of *Kp2* in  
314 the presence of CCCP (Table 3). No such change was encountered for other isolates either with  
315 CCCP or with any other EPI. CCCP is a nonspecific EPI and acts on the energy source of several  
316 efflux pumps as well as on other membrane proteins. Decreased MIC of colistin in the presence of  
317 CCCP might indicate a probable role of efflux pump in colistin resistance in *Kp2* [17, 34].

318 RT-PCR showed overexpression of regulators and efflux pump genes viz. *ramA* (5.5-fold),  
319 *soxS* (2.7-fold), *acrB* (4.5-fold), and *tolC* (2-fold), in *Kp2* only when compared with the colistin-  
320 susceptible *K. pneumoniae* (EN5219) (Table 3) and *Kp1*, *Kp3*, and *Kp4*. This further substantiated  
321 the change in MIC value for colistin in presence of CCCP in *Kp2*. This indicated role of an active  
322 AcrAB-TolC efflux pump conferring colistin resistance in *Kp2*. Padilla *et al.* (2010) also reported  
323 the presence of an energy-dependent efflux of colistin by efflux pump gene, *acrB* [17] similar to our  
324 observation.

325 Overexpression of *soxRS* has been found to induce colistin hetero-resistance in *Enterobacter*  
326 sp. [35] which triggered upregulation of *acrA* and *tolC*. Though upregulation of *soxS* has been found  
327 in the study, yet no change in the sequences of *soxS* and its regulator, *soxR* have been noticed among  
328 the strains. As there was overexpression of *acrB*, *tolC* and its regulators *ramA*, their sequences were



analyzed but no differences were noticed in the WGS data of the four isolates. Since, *ramA* is the global regulator of *acrB*, the promoter sequences of *ramA*, *soxS*, *acrB*, and *tolC* were also assessed with no differences to be identified from the genomic data. Further, sequences of local repressor of *ramA*, *acrA*, and *acrB*, (*ramR*, and *acrR*, respectively) were investigated, but no variation was noticed in these gene sequences. Similar observations for the overexpressed AcrAB-TolC pump and *ramA* have been made in other studies, with no genetic background to substantiate such findings, thereby indicating presence of an alternate mechanism regulating expression of these genes [17,36]. *ramA* mediated alteration of lipid A moiety in *K. pneumoniae* has also been reported to result in altered susceptibility to colistin [37]. Hence, increased *ramA* levels can also mediate LPS alterations, which along with the AcrAB-TolC likely contributed towards increased survival in presence of colistin. In this study also, *Kp2* had increased expression of *ramA*. Therefore, it is likely that a functional AcrAB-TolC pump and *ramA* are crucial in mediating decreased susceptibility towards colistin in *Kp2*.

*KpnEF* can efficiently efflux colistin whereas *KpnGH* acts as an intrinsic resistance determinant in *K. pneumoniae* [19, 20]. However, in our study, the four isolates showed similar level of expression for both the SMR and MFS type efflux pumps implying no role of these efflux pumps towards colistin resistance.

Apart from these, presence of a new transmembrane protein, *Enterobacter* colistin resistance gene (*ecr*) corresponding to colistin resistance has been reported [38]. But no such homologue was found to be present in the genome of *Kp2*.

Additionally, resistance to colistin is also attributed to change in porin profile among Enterobacteriaceae [39]. All the isolates showed similar pattern of porins, loss of *OmpK35* but intact *OmpK36*. With no observed difference in the profile of porins in the colistin-resistant and

352 susceptible isolates, it was concluded that OMPs had no role to play towards colistin resistance in  
353 this study.

#### 354 **4. Conclusion-**

355 This study reports the emergence of KPC-2 in ST147 *K. pneumoniae* isolated from septicemic  
356 neonates. The strains showed 100% mutual identities and possessed a repertoire of resistance and  
357 virulence determinants. A difference in colistin susceptibility among the indistinguishable strains  
358 was attributed to AcrAB-TolC pump overexpression. The present study is probably the first to report  
359 such differences in colistin susceptibility for clonal *K. pneumoniae* isolates and similar occurrence  
360 has never been demonstrated in clinical isolates. WGS data did not indicate any change in the  
361 homologs known to be involved in the expression of the AcrAB-TolC pump system. This  
362 differential expression may be attributed to hitherto unknown regulatory mechanism of AcrAB-TolC  
363 pump. This phenomenon of variation in expression of genetically identical pump in isolates which  
364 are nearly similar, enhances our perspective on the versatility of resistance mechanisms in gram-  
365 negative bacteria.

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#### 372 **Accession numbers-**

373 All genomes sequences have been submitted to NCBI database with SRA accession number  
374 SRP136309, BioProject accession number PRJNA445372 and GenBank accession numbers  
375 PYWD000000000.1 (EN5226), QCZB000000000.1 (EN5261), QCZC000000000.1 (EN5269), and  
376 PYWE000000000.1 (EN5271).

377

378

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386 **Ethic Approval:** The study protocol was approved by the Institutional Ethics Committee of the  
387 ICMR-National Institute of Cholera and Enteric Diseases (No. A-1/2016-IEC). Patient information  
388 was anonymized and de-identified prior to analysis.

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519 **Figure Legend**

520 **Fig. 1.** Schematic representation of Tn4401b bearing *bla<sub>KPC-2</sub>*.

521 Genes and their corresponding transcription orientations are indicated by horizontal arrows.  
522 Tn4401b is delimited by two inverted repeat sequences, IRR and IRL (grey triangles). Small open  
523 triangles represent the inverted repeats of IS*Kpn6* and IS*Kpn7*.

524

525 **Fig. 2.** Diagrammatic representation of class 1 integron found in KPC-2 bearing isolates.

526 *arr-2* (ADP-ribosyl transferase), *S.ma.I2* (group IIc intron), *qacEΔ1* (quaternary ammonium  
527 compound resistance protein), *sulII* (sulphonamide resistant dihydropteroate synthase), **orf5** (an open  
528 reading frame of unknown function).

529

530 **Fig. 3.** BRIG analysis of KPC-2-producing *K. pneumoniae*.

531 Comparative genome map of KPC-2-producing *K. pneumoniae* using *Klebsiella pneumoniae* subsp.  
532 *pneumoniae* HS11286 as a reference. From the inside out, circle 1 represents the mean centered G+C  
533 content; circle 2 shows GC skew calculated as  $(G - C) / (G + C)$ ; circle 3 to 6 represent the genome  
534 sequence similarity of EN5269 (*Kp3*), EN5226 (*Kp1*), EN5271 (*Kp4*), and EN5261 (*Kp2*) based on  
535 color gradient against the reference genome. The color gradients indicate the degree matching in the  
536 BLAST result for a shared region, as given to the top right of the ring.

537

538 **Fig. 4.** Molecular typing and relatedness of KPC-2-producing *K. pneumoniae* isolates. (A)  
539 Phylogenetic tree analysis among the *bla<sub>KPC-2</sub>* containing isolates under study using iTOL. Branch  
540 length has been depicted showing evolutionary differences between the isolates. *Kp1* & *Kp3* are  
541 closely related so as *Kp2* & *Kp4*. (B) PFGE of *XbaI*-digested genomic DNA of KPC-2-producing *K.*

542 *pneumoniae*, isolated from blood of 4 septicemic neonates. Lane 1: *Kp2*; lane 2: *Kp1*; lane 3: *Kp3*;  
543 lane 4: *Kp4*, and lanes M: *Salmonella* serotype *Braenderup H9812* as reference standard.